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Report

Expression Partitioning between Genes Duplicated by Polyploidy under Abiotic Stress and during Organ Development

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Summary

Allopolyploidy has been a prominent mode of speciation and a recurrent process during plant evolution and has contributed greatly to the large number of duplicated genes in plant genomes [1–4]. Polyploidy often leads to changes in genome organization and gene expression [5–9]. The expression of genes that are duplicated by polyploidy (termed homeologs) can be partitioned between the duplicates so that one copy is expressed and functions only in some organs and the other copy is expressed only in other organs, indicative of subfunctionalization [10]. To determine how homeologous-gene expression patterns change during organ development and in response to abiotic stress conditions, we have examined expression of the alcohol dehydrogenase gene *AdhA* in allopolyploid cotton (*Gossypium hirsutum*). Expression ratios of the two homeologs vary considerably during the development of organs from seedlings and fruits. Abiotic stress treatments, including cold, dark, and water submersion, altered homeologous-gene expression. Most notably, only one copy is expressed in hypocotyls during a water-submersion treatment, and only the other copy is expressed during cold stress. These results imply that subfunctionalization of genes duplicated by polyploidy has occurred in response to abiotic stress conditions. Partitioning of duplicate gene expression in response to environmental stress may lead to duplicate gene retention during subsequent evolution.

Results and Discussion

Polyploidization in *Gossypium* between an A genome diploid species and a D genome diploid species approximately 1.5 mya [11] created the AD allotetraploid lineage that includes the agronomically important *G. hirsutum* and *G. barbadense*. Previous studies of homeologous gene expression in polyploid cotton have revealed considerable organ-specific expression biases and silencing of several genes in both natural polyploids and synthetic neopolyploids [10, 12]. Most notably, the two homeologous copies of certain genes are expressed in an organ-specific manner such that only the D_t copy (derived from the D genome parent) is expressed in some organs, whereas only the A_t copy (derived from the A genome parent) is expressed in other organs. Partitioning of expression between the two duplicates, such that only one copy is expressed in some organs and only the other copy is expressed in other organs, indicates subfunctionalization [13] and suggests that the two duplicated copies will be retained. The organ-specific expression and silencing patterns of duplicated genes in polyploid cotton suggest that homeologous-gene expression ratios could change during organ development or in response to stress conditions. For example, there could be expression of both copies at some stages during development and only one copy at others. It is possible that one copy might function better and be more highly expressed in response to certain stresses or at certain developmental stages. In this study, we have used duplicated *AdhA* genes in polyploid cotton to study expression patterns of homeologous genes during organ development and in response to abiotic stress conditions. *AdhA* genes were chosen because they show organ-specific partitioning of expression of the two homeologous copies in flower organs [10].

Extensive Variation in Homeologous-Gene Expression Patterns during Organ Development

To study *AdhA* expression variation during seedling development, we assayed expression ratios of *AdhA* homeologs at eight different time points during the development of hypocotyls, cotyledons, and roots of *G. hirsutum*. To determine the expression ratio between the A_t and D_t homeologs, derived from the A and D parental genomes, we used a single nucleotide primer-extension assay (see Experimental Procedures in the Supplemental Data available online). Allelic studies in humans and mice with the assay have indicated that the technique provides a quantitative measure of allelic expression or presence (e.g., [14–18]). The expression ratios of the A_t and D_t homeologs varied greatly during the development of seedling organs (Figure 1). In cotyledons, the A_t/D_t expression ratio at 6 days was approximately 2:1 but then declined to a strong bias toward D_t at 11 days. However, by 32 days the ratio was again approximately 2:1, and by 38 days there was a strong bias toward A_t. In contrast, in hypocotyls at 6 days there was only expression of A_t (as determined by direct sequencing of RT-PCR products), but then the A_t/D_t expression ratio changed to being approximately equal at 14 days, and it stayed relatively constant through 38 days. Expression levels in roots were less variable with only one comparison being statistically significant. We also assayed total levels of expression of both homeologs by real-time qRT-PCR (Figure S1). There were no obvious correlations between total expression levels and ratios of homeologous-gene expression.

A previous study of *AdhA* expression in ovules examined expression at two time points during development

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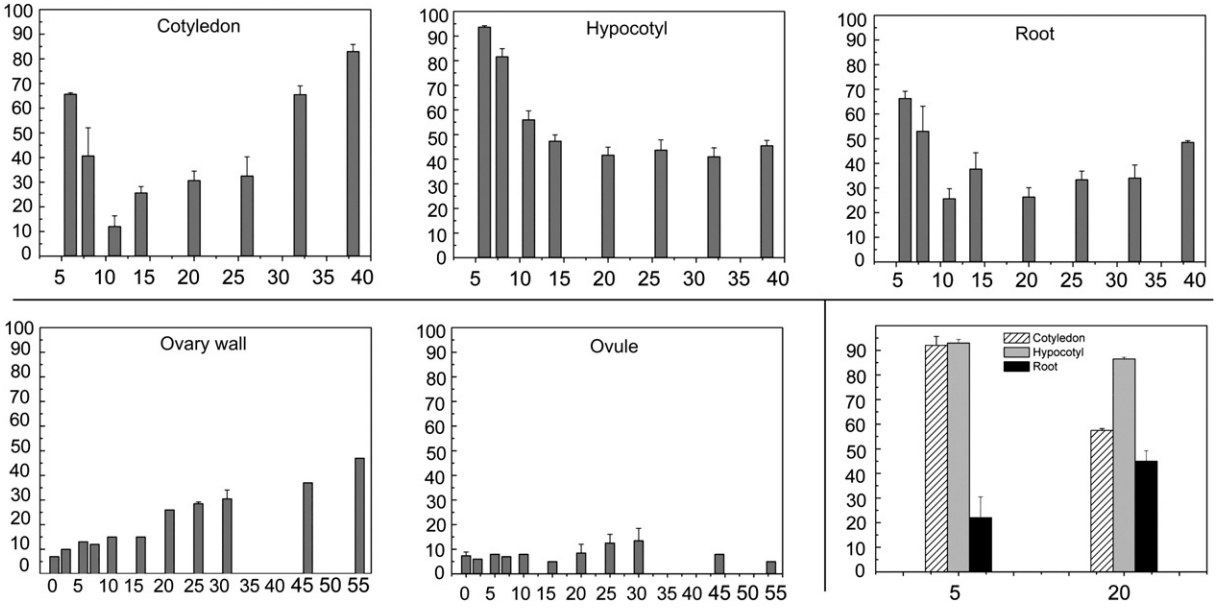

Figure 1. Ratios of A_t/D_t Expression Vary during Organ Development

Graphs showing the percentage of transcripts derived from the A_t homeolog (y axis) in seedling organs and fruit organs (ovary wall and ovule) from *G. hirsutum*. The x axis indicates the days after planting for seedlings or days after flower opening for ovules and ovary walls. Error bars represent SDs among replicates. Some time points for ovary walls and ovules had a single replicate, and, hence, no error bars. One-way ANOVAs were used so that significant differences at successive developmental time points for the seedling data could be tested. For the cotyledons, the 6–8 day, 8–11 day, and 26–32 day comparisons were significantly different (p = 0.002, 0.001, and 0.001, respectively). For the hypocotyls, the 6–8 day and 8–11 day comparisons were significantly different (p = 0.005 and 0.000, respectively). For the roots, only the 8–11 day comparison was significant (p = 0.000). The lower-right panel contains a graph showing the percentage of transcripts derived from the A_t homeolog in seedling organs from a synthetic neopolyploid at the following two time points: 5 days and 20 days. All organs are shown on the same graph. One-way ANOVA tests indicated that the 5–20 day comparisons for cotyledons and roots were significant (p = 0.0283 and 0.001, respectively), but expression in the hypocotyls at the two time points was not significantly different.

[10]. Here, we have examined *AdhA* expression at 11 time points during the development of ovules and ovary walls in *G. hirsutum*. In ovary walls on the day of flower opening, there was only expression of the D_t copy (as determined by direct sequencing of RT-PCR products), and the ratio of A_t/D_t expression increased relatively steadily throughout ovary wall development to approximately 1:1 at 53 days after flower opening (Figure 1). Thus, like seedling organs, ovary walls show expression ratios that change during organ development. The ovules showed only expression of the D_t copy on the day of flower opening, but, in contrast to the ovary walls, the ratio of A_t/D_t expression increased only slightly during development to 53 days after flower opening. The expression levels at 10 days are the same but differ at 20 days from the previous study [10]; the difference could be due to removing most of the fiber tissue from the ovules in the current study, growing the plants at a different location, or other environmental factors. Total levels of *AdhA* expression in ovary walls, as measured by real-time qRT-PCR (Figure S1), did not show correlations with ratios of homeologous-gene expression levels, as was the case with seedling organs.

Synthetic cotton neopolyploids can show organ-specific expression of homeologous genes [10, 12]. To determine whether homeologous-gene expression ratios can change during organ development in neopolyploids, we examined *AdhA* expression in a synthetic cotton allopolyploid (see Supplemental Experimental Procedures) at two time points per organ. In cotyledons, there was only expression of the A_t copy at 5 days (as determined by direct sequencing of RT-PCR products), but at 20 days, both copies were expressed at an approximately 60:40 ratio (Figure 1, lower-right panel). Roots showed a different pattern with a strong D_t bias at 5 days and then relatively equal A_t/D_t expression at 20 days. These results show that homeologous-gene expression ratios can change considerably during organ development in a neopolyploid and that the phenomenon is not restricted to natural allopolyploids. For the most part, there was no similarly in A_t/D_t expression ratios in *G. hirsutum* and the neopolyploid plants, suggesting that mutations that are unique to *G. hirsutum* in regulatory regions or to the neopolyploid may play a role in regulating expression levels.

The results from the studies of *AdhA* expression during seedling and ovary wall development indicate that relative expression levels of homeologous genes can change considerably during organ development and that only one copy can be expressed during some developmental stages, whereas both copies are expressed at other stages. Previous studies that examined homeologous-gene expression in multiple organs of polyploids almost exclusively used a single developmental stage of each organ [10, 12, 19–21]. Thus, the amount of organ-specific variation in expression for other homeologous genes and other plants, if other developmental stages were to be assayed, is likely to be considerably greater than previously reported.

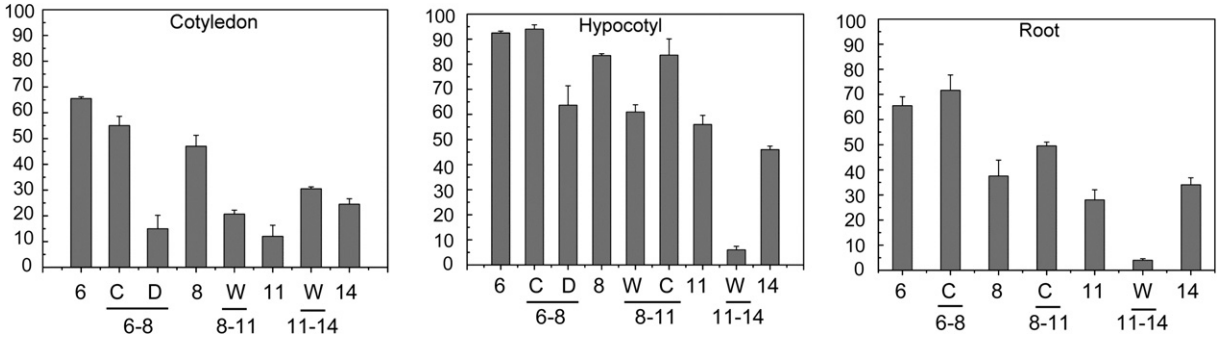

Figure 2. Ratios of A_t/D_t Expression Vary under Stress Conditions

Graphs showing the percentage of transcripts derived from the A_t homeolog (y axis) in seedling organs of *G. hirsutum* at different days after planting. "C" indicates cold stress, "D" indicates dark stress, and "W" indicates water submersion. Error bars represent SDs among replicates. One-way ANOVA tests indicated that the following treatments were significantly different than untreated: cotyledons: dark 6–8 days ($p = 0.008$); hypocotyls: cold 6–8 days, dark 6–8 days, cold 8–11 days, and water submersion 11–14 days ($p = 0.004$, 0.021, 0.002, and 0.000, respectively); roots: cold 8–11 days and water submersion 11–14 days ($p = 0.001$ and 0.001, respectively). All other treatments did not result in a statistically significant difference.

Partitioning of Homeologous-Gene Expression in Response to Abiotic Stress

Expression of alcohol dehydrogenase genes in plants has been shown to increase in response to cold temperature, anaerobic stress, and water-logging conditions, suggesting that ADH enzymes play a role in stress survival (reviewed in [22]). We hypothesized that a gene that is responsive to certain stress conditions might show differences in homeologous expression in response to those conditions. To determine whether abiotic stress affects homeologous-gene expression, we performed water-submersion, cold stress, and dark stress experiments by using *G. hirsutum* seedlings at different stages of development. Water-submersion experiments included a 4 day submersion in distilled water at room temperature at two different developmental stages. Water submersion had the most dramatic effect during the 11–14 day submersion experiment. Expression ratios of the A_t and D_t homeologs in untreated plants were similar at the beginning and end of the experiment in hypocotyls (roughly equal) and roots (an approximately 1:2 ratio), as shown in Figure 2. However, water submersion caused silencing of the A_t copy in both hypocotyls and roots (Figure 3). In contrast, the A_t/D_t expression ratio in cotyledons was higher in treated plants than in untreated plants. Cotyledons and hypocotyls from the 8–11 day water submersion treatment also showed different A_t/D_t expression ratios compared with untreated plants but no homeologous-gene silencing (Figure 2). The changes in gene expression compared with control plants in these experiments could have been caused by water logging, by the less aerobic environment, or by both factors.

Cold stress experiments included a 3 or 4 day exposure at 4°C at two different stages of seedling development. Hypocotyls from the 6–8 day cold treatment showed silencing of the D_t copy (Figure 3). Untreated plants displayed silencing of the D_t copy at 6 days but coexpression at 8 days, albeit with a strong bias toward A_t (Figure 2); thus, there was a difference between cold-treated and untreated plants at the end of the experiment. Cotyledons and roots also showed different A_t and D_t expression ratios compared with untreated plants but no homeologous-gene silencing. A second cold stress treatment was done from 8–11 days. A_t and D_t expression ratios increased in roots of cold-treated plants compared with untreated plants, but expression in hypocotyls remained about the same as in the normal plants at 8 days. The results from the cold stress experiments indicate that cold stress affected homeologous expression of *AdhA*, but overall the effects were not as great as those for water-submersion stress.

Dark stress experiments included a 3 day dark treatment at one stage of seedling development. Cotyledons from plants in the 6–8 day dark treatment showed a strong bias toward the D_t homeolog of *AdhA* (Figure 2), representing a considerable difference from those in untreated plants. Hypocotyls also showed a change in the A_t/D_t expression ratio in plants after the stress treatments compared with those in untreated plants, although the change was not as dramatic as in cotyledons. In addition to assaying the A_t and D_t expression ratios, we also assayed total levels of *AdhA* expression for all of the stress conditions (Figure S2). As was the case with normal conditions, there were no obvious correlations with the ratios of homeologous-gene expression.

When considered together, the results from the water-submersion treatment (A_t silencing in hypocotyls and roots during the 11–14 day treatment) and the cold stress treatment (D_t silencing in hypocotyls during the 6–8 day treatment) show reciprocal silencing of the A_t and D_t copies of *AdhA* (Figure 3). To determine whether the gene is expressed in the diploid progenitor species under the same stress conditions, we performed stress treatments on seedlings from the diploids and assayed *AdhA* expression. *AdhA* is expressed in both diploid species under the stress conditions (Figure S3). The stress experiments show that expression of the two *AdhA* homeologs has been partitioned in response to abiotic stress conditions, suggesting subfunctionalization, and that both copies will be retained during subsequent evolution.

The differing expression patterns of the A_t and D_t copies of *AdhA* indicate that the genes are differently regulated. One possibility for explaining the differential

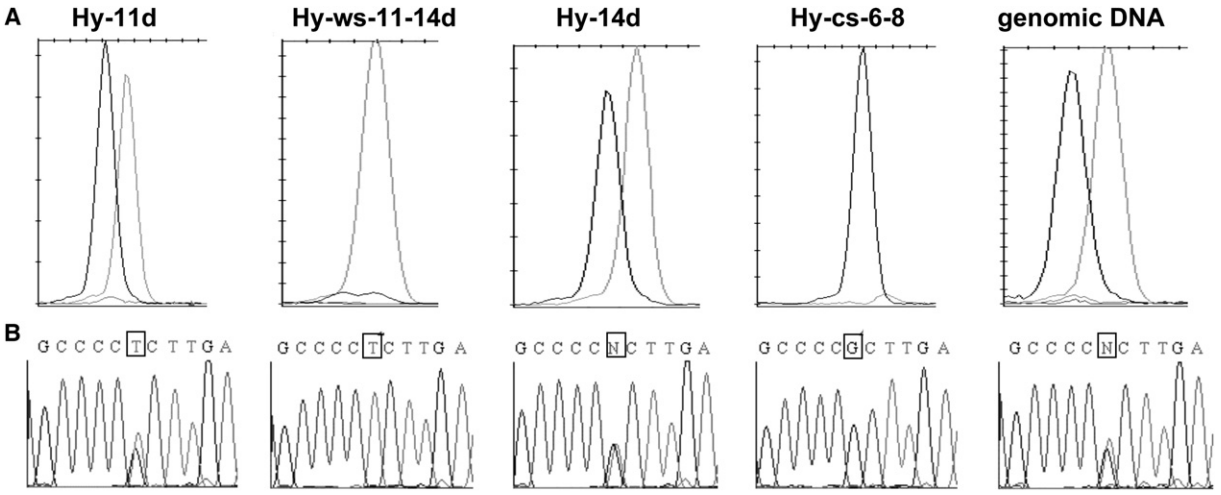

Figure 3. Reciprocal Silencing of *AdhA* Homeologs under Stress Conditions

(A) Chromatograms from single-nucleotide primer-extension analyses. The darker peaks are from the A_t homeolog, and the lighter peaks are from the D_t homeolog. Experiments and organs are indicated above (A).

(B) Chromatograms from direct sequencing of RT-PCR products. A single nucleotide polymorphism between the A_t and D_t copies is boxed. Other polymorphisms between the A_t and D_t copies (not shown) also were evaluated. Abbreviations are used as follows: Hy, hypocotyls; ws, water submersion; and cs, cold stress.

regulation is that mutations have occurred in the *cis*-regulatory elements in one copy or the other, as predicted by the duplication-degeneration-complementation model [23]. We sequenced upstream of the start codon from several polyploid and diploid species and identified several mutations that occurred after polyploidy (Figure S5). It was unclear whether any of the mutations occurred in bone fide regulatory elements.

Subfunctionalization and the Evolution of Duplicate Genes

Subfunctionalization, the partitioning of ancestral function or expression patterns between duplicated genes so that both are retained, is generally regarded as being one of the major outcomes for duplicated genes (e.g., [13, 23–28]). There have been several reported examples of partitioning of ancestral expression patterns between duplicated genes in different plant organs [10, 29–33] and in animals (e.g., [23, 34]). Other studies have provided evidence for partitioning of function between duplicated genes (e.g., [35, 36]). In plants, two notable recent reports of functional partitioning between duplicates are *PhyB1* and *PhyB2* in maize [37] and *OsMADS3* and *OsMADS58* in rice [38]. A study of polygalacturonase-inhibiting proteins in beans inferred subfunctionalizaiton in response to biotic stress on the basis of certain genes that have unique functions in the family [39]; however, the results could instead be interpreted as potential cases of neofunctionalization, so the nature of the functional changes is not clear.

The subfunctionalization of *AdhA* in *G. hirsutum* involves partitioning of expression among organ types and in response to abiotic stress. It is not clear which occurred first or whether expression partitioning in response to stress was a byproduct of organ-specific expression partitioning, or vice versa. There may well be other abiotic stresses that are not examined in this study and that result in silencing of one copy or the other.

This is the first report of subfunctionalization of duplicated genes in an allopolyploid in response to environmental stress. How general might the phenomenon be? Previous studies of homeologous-gene expression in polyploids have not examined expression in response to stress conditions, but we predict that the phenomenon will be detectable, perhaps even relatively common, for other genes in other polyploids plants. It would be interesting in the future to examine expression of sets of homeologous genes in allopolyploids in response to various abiotic and biotic stresses, especially those genes for which the total expression level increases or decreases in response to stress conditions. A recent bioinformatics study of genes duplicated by an ancient polyploidy during the evolutionary history in the *Arabidopsis* lineage indicated that duplicate gene expression diverges more under environmental stress conditions than in different organs and during development [40], suggesting subfunctionalization, neofunctionalization, or subneofunctionalization (a process proposed by [41]) of some of those anciently duplicated genes.

Subfunctionalization of duplicated genes in response to stress suggests that some of the thousands of genes duplicated by polyploidy in plant genomes are preserved because their expression has been partitioned in response to environmental stress. Neofunctionalization, the gain of new expression patterns and functions, in response to stress conditions also may help account for some of the retained duplicates. Neofunctionalization and subfunctionalization of some duplicated genes might enable plants to better cope with stresses in their natural environments and help contribute to the evolutionary success of polyploids.

Supplemental Data

Experimental Procedures, five figures, and one table are available at http://www.current-biology.com/cgi/content/full/17/19/1669/DC1/.

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Accession Numbers

GenBank numbers for the sequences determined in this study are EF591131–EF591141.